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<p>(54) Title: OLIGONUCLEOTIDE PROBES FOR DETECTION OF PERIODONTAL PATHOGENS</p> <p>(57) Abstract</p> <p>This invention relates to compositions of oligonucleotide probes for use in the detection of bacteria associated with medical disorders of the human mouth, wherein said probes consist essentially of a segment of nucleic acid capable of selectively hybridizing under hybridizing conditions, to the 16S or 23S ribosomal RNA [rRNA] of said bacteria. Methods for detection, as well as diagnostic kits for the assay of these bacterium, are also disclosed.</p>		

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OLIGONUCLEOTIDE PROBES FOR  
DETECTION OF PERIODONTAL PATHOGENS

BACKGROUND OF THE INVENTION

5    Technical Field of the Invention

          This invention relates to compositions of oligonucleotide probes for use in the detection of bacteria associated with medical disorders of the human mouth, wherein said probes consist essentially of a  
10   segment of nucleic acid capable of selectively hybridizing under hybridizing conditions, to the 16S or 23S ribosomal RNA [rRNA] of said bacteria. Methods for detection, as well as diagnostic kits for the assay of these bacteria, are also disclosed.

15           Substantial evidence exists implicating plaque bacteria in the etiology of human periodontal disease, particularly gingivitis and periodontitis. As these diseases have been studied, increasingly sophisticated methods for detection, quantitation, and identification  
20   of specific oral bacteria and other infectious viruses and microorganisms have been developed. These improved methods have led to the identification of specific pathogenic bacteria playing a major role in the etiology of periodontal disease.

25           Human periodontal diseases constitute a major health-care problem in the United States. While epidemiologic surveys suggest that there may be a reduction of the proportion of Americans with gingivitis in recent years, the number of patients having some stage of the  
30   disease remains high. Furthermore, the proportion of adults, aged 18-79 years, with periodontal pockets appears to be relatively unchanged.

          In the early 1970's, roughly 30 percent of Americans had significant periodontal disease problems by  
35   age 50, and by age 60, between 30 and 40 percent of all Americans had lost all of their teeth, primarily due to

periodontal disease. At present, the exact proportion of persons with severe periodontal disease is unknown, but recent estimates are that 10-20 percent of the overall American population has periodontal disease serious enough to result in tooth loss. Coincidental with the disease is a significant relationship between the onset and course of the disease, and identifiable (suspect) pathogenic bacterial species.

Although the precise complex mechanisms involved in the pathogenesis of human periodontitis are still unclear, there is wide agreement that bacteria that colonize the gingival crevice area around the teeth are of primary etiologic importance. Within the past decade, researchers at several academic institutions have demonstrated that of the large number of bacterial genera and species found in the human periodontal crevice or pocket (ca. 256 species), a relatively limited number of these appear to be consistently associated with periodontal disease. These microorganisms have been designated "suspect periodontal pathogens." Of particular interest are the microorganisms shown in Table 1.

#### INFORMATION DISCLOSURE

The detection and characterization of the periodontal pathogenic bacteria present in a given periodontal pocket site are, by current methods, both time-consuming and expensive (e.g., microscopic, cultural, gas chromatographic and metabolic product analysis). Furthermore, highly trained individuals, with sophisticated laboratory equipment, are required. Most of the published studies on the microbiology of human periodontal diseases have been compromised by the enormous workload that must be accomplished to isolate and characterize individual members of the microflora at each periodontal site. As a result, the number of sites sampled per patient and the number of patients examined are limited. Clearly, the present methods do not allow

routine testing of periodontal patients for the presence of pathogenic bacteria.

Immunoassays are reported as an alternative to the above detection methodologies. Fluorescent monoclonal antibodies to *Actinobacillus actinomycetemcomitans* (A.a.), *Bacteroides gingivalis* (B.g.), and *Bacteroides intermedius* (B.i.) have been developed. Chen, P., et al., The Use Of Monoclonal Antibodies To Detect *Bacteroides gingivalis* In Biological Samples, *Infection and Immunity*, 54:798-803 (1986); Gmur, R., et al., Quantitative Monitoring Of The Periodontal Pocket Flora With Monoclonal Antibodies, *Journal of Dental Research* Vol. 66:120 (1987, abstract); Fine, D.H. and Mandel, I.D. Indicators Of Periodontal Disease Activity: An Evaluation, *J. Clin. Periodontol*, 13:533-546 (1986). These antibodies can be used directly on plaque smears on microscope slides. The testing requires manual counting of fluorescent bacteria on individual microscope slides, a procedure which is very labor-intensive and requires an expensive fluorescent microscope.

The potential for using nucleic acid hybridization technology to detect pathogenic bacteria is known and, more specifically, the use of rRNA for hybridization assays is known (Canadian Patent No. 1,215,904 and Oligonucleotide Probes Complementary to Variable Regions of Ribosomal RNA Discriminate between *Mycoplasma* Species. *J. General Microbiology* (1987) 133, 1969-1974. U.B. Gobel, A. Geiser, E.J. Stanbridge). Whole genomic DNA probes have reportedly been used to identify periodontal pathogens (EP 199,439), but these methods do not possess the hybridization specificity of the disclosed invention. For example, fragments of A.a., B.g., or B.i. genomic DNA have been cloned into the pSP64 transcription vector. Chen, M., et al., Development Of DNA Probes For Oral Microorganisms, Assoc. Adv. Dental Res. Meetings, Wash., D.C. (1986); Savitt, E., et al., Detection of *A. actinomycetemcomitans* In Plaque By RNA Probes, Assoc.

Adv. Dental Res. Meetings, Wash., D.C. (1986). The pSP64 vector is used to produce A.a, B.g., or B.i. RNA transcripts. A pSP64-A.a. probe was reported to be specific when tested against pure cultures or plaque samples. However, pSP64-B.g. and pSP64-B.i. probes cross-hybridized slightly with some non-B.g. and non-B.i. bacteria, respectively. Since non-pathogenic Bacteroides species are abundant in the subgingival crevice, these cross-hybridizations may cause false positive identifications.

Each of the above methods of detecting periodontal pathogens lacks one or more of the following criteria necessary for a routine diagnostic assay: 1) cost (inexpensive); 2) batch capability; 3) speed (hours, not days or weeks); 4) sensitivity; 5) specificity; 6) multiple pathogen identification in one test; 7) simplicity; and 8) ability to detect both viable and non-viable organisms. Consequently, there is a need in the art for alternate detection methodologies.

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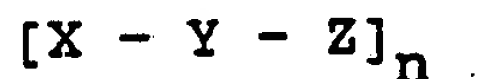
## SUMMARY OF THE INVENTION

The present invention is directed to compositions of oligonucleotide probes for the detection of bacteria associated with human oral medical disorders, wherein said probes comprise a segment of nucleic acid capable of selectively hybridizing, under hybridizing conditions, to hypervariable regions of ribosomal RNA of the bacteria with the provision that any additional nucleotides covalently bound to said segment do not hybridize under said conditions to nucleic acids of bacteria commonly found in the human mouth. Functionally, these probes are able to hybridize to unique portions of the bacterial rRNA or corresponding genomic DNA permitting ready detection and identification of such bacteria without cross-reacting with other bacterial species. In addition, compositions of oligonucleotide probes directed to conserved regions of 16S and 23S ribosomal RNA are



described, wherein said probes can be utilized as specific probes for ribosomal RNA or the corresponding genomic sequence, and can be used for sequencing of ribosomal nucleic acids.

5 This invention is also directed to a probe of the formula:



wherein:

10 a) X is a sequence of 0 to 100 nucleotides or nucleotide analogs that are non-homologous to conserved or non-conserved regions of bacterial nucleic acid found in bacteria inhabiting human mouths;

15 b) Y is a sequence of 10 to 100 nucleotides or nucleotide analogs that are capable of hybridizing under hybridizing conditions to hypervariable regions of the ribosomal RNA of bacteria inhabiting human mouths, such that Y may also comprise subsequences that are capable of hybridizing under hybridizing conditions to only one species of said bacteria, to two species of said bacteria  
20 or to three species of said bacteria;

c) Z is a sequence of nucleotides the same as or different from X, such that nucleotides or nucleotide analogs are non-homologous to conserved or non-conserved regions of nucleic acid of bacteria found inhabiting  
25 human mouths; and

d) n is 1-500, or more and, where n is greater than 1, Y can be the same or different sequences of nucleotides having said hybridization capability. The probe can be free or contained within a vector sequence  
30 (e.g., plasmids or Single Stranded DNA).

The following bacteria are of particular interest, because they have been found in association with periodontal disease: Actinobacillus (ex. Haemophilus) actinomycetemcomitans; Bacteroides  
35 gingivalis; Bacteroides intermedius Type 1; Bacteroides intermedius Type 2; Eikenella corrodens; Bacteroides forsythus; Fusobacterium nucleatum; Fusobacterium perio-

donticum; Streptococcus intermedius; Wolinella recta. This invention discloses compositions comprising polynucleotide probes able to hybridize to rRNA of these bacteria, especially the hypervariable regions of the 16S  
5 and 23S rRNA.

It is preferred that each specific probe hybridize to the rRNA of only one microbial species or type. Especially preferred are sequences complementary to the hypervariable regions of the 16S rRNA, as offered  
10 in Table 1, but these are not intended to be limiting. Alternative sequences are readily ascertainable using the sequencing methods disclosed herein. For example, sequences for the hypervariable regions from the 23S ribosomal RNA could be determined using the sequencing  
15 primers shown in Table 2. Using the methods disclosed herein, one of skill in the art could readily obtain alternative rRNA sequences with suitable specificity for use in this invention.

The nucleic acid sequence of the claimed probes  
20 include synthetically derived or recombinant nucleic acid sequences which have sufficient identity with the claimed sequences that they substantially hybridize with regions complementary to the claimed probes. By "substantially", it is meant that under standard hybridization conditions  
25 of moderate stringency, percent hybridization can be shown to exceed 50% of the hybridization between perfectly complementary nucleic acid fragments.

By "compositions", it is meant that probes complementary to bacterial rRNA may be in a pure state or in  
30 combination with other probes. In addition, the probes may be in combination with salts or buffers, and may be in a dried state, in an alcohol solution as a precipitate, or in an aqueous solution.

The phrase "and combinations thereof," in the  
35 context of bacterial groups, refers to compositions of probes designed to detect one or more of the bacterial species stated. The probes may be a mixture of different



probes capable of detecting a single species or two or more species, a mixture of different probes wherein the probes are each able to detect one or more species, or a homogeneous composition of a single probe. In the context of oligonucleotide sequences, the phrase "and combinations thereof" refers to a composition of copies of a single probe that may contain as a part of the probe one or more copies of a single oligonucleotide sequence or a mixture of the given sequences, or a mixture of probes that may contain as a part of the probes single or multiple copies of the given oligonucleotide sequences.

The terms oligonucleotide or polynucleotide probes are meant to include both double stranded and single stranded DNA or RNA. The terms also refer to synthetically or recombinantly derived sequences essentially free of non-nucleic acid contamination.

In addition to composition claims, methods are disclosed for the detection of a microbial cell associated with a medical disorder, in a sample obtained from the mouth of a human patient. These methods comprise the steps of: lysing the microbial cells to free the ribosomal RNA; contacting said ribosomal RNA, under hybridizing conditions, with polynucleotide probes capable of selectively hybridizing to the hypervariable regions of the ribosomal RNA of said microbial cell; and detecting hybridization complexes as an indication of the presence of the microbial cell in the sample. More specifically, there is disclosed herein the above method, where the bacteria detected are selected from the above list. Even more specifically, there is disclosed herein a method, as described above, in which the samples are assayed using at least two different groups of probes where each group is capable of hybridizing to a different hypervariable region of the ribosomal RNA of a single bacterium.

In addition to the above compositions and methods, there are disclosed herein diagnostic kits for

use in determining the presence of bacteria which comprise a synthetic oligonucleotide probe complementary to hypervariable regions of the ribosomal RNA of a microbial cell associated with a human oral medical disorder.

5           The taxonomy of these bacteria is not static. Type cultures are available from the American Type Culture Collection (ATCC), Rockville, Maryland. As other bacteria are identified or other subtypes are differentiated from the above list, the disclosed invention  
10 readily provides methods for preparing probes of sufficient specificity for use in the detection and identification of these bacteria.

          A composition of polynucleotide probes is also claimed wherein said probes comprise a segment of nucleic  
15 acid capable of selectively hybridizing to regions of ribosomal RNA of the bacteria having minimal secondary or tertiary interactions with adjacent nucleotides (open regions), said probes substantially binding only to open regions. By "substantially binding" it is meant that the  
20 probes do not comprise significant sequences that bind to regions that are available for hybridization only after heating, that is, regions with significant secondary and tertiary structure (closed regions). In practical terms, such probes will generally not comprise any more than 10  
25 flanking nucleotides (either 5' or 3') which would bind to closed regions.

          More specifically, compositions of polynucleotide probes complementary to open regions are claimed that are complementary to both hypervariable and conserved regions  
30 of bacterial rRNA.

          A composition of polynucleotide probes is also claimed that hybridize to closed, conserved regions of bacterial rRNA.

          Finally, methods are disclosed for the detection  
35 of microbial cells in a sample obtained from the mouth of a human patient. The method comprising the steps of: lysing the microbial cells to free the ribosomal RNA;

contacting said ribosomal RNA, under hybridizing conditions, with polynucleotide probes capable of selectively hybridizing to open regions of the ribosomal RNA of said microbial cell; and detecting hybridization complexes as an indication of the presence of the microbial cell in the sample. More specifically, there is disclosed herein the above method, where the bacteria detected are selected from the above list. Even more specifically, there is disclosed herein a method, as described above, in which the samples are assayed using at least two different groups of probes where each group is capable of hybridizing to a different conserved region of the ribosomal RNA of a single bacterium.

#### DETAILED DESCRIPTION

This invention relates to the development of species specific nucleic acid probes complementary to the rRNA of species of oral bacteria associated with periodontal disease and other bacterial mediated diseases of the mouth. These probes are superior to probes complementary to genomic sequences because of their specificity and sensitivity. These probes have been selected for their ability to avoid cross-hybridizing with non-pathogenic bacterial species. Their superior properties are due to the ability to hybridize exclusively to hypervariable sequences of rRNA, which appear in hundreds to thousands of copies throughout each bacterial cell. The hypervariable regions of rRNA are sequences which are peculiar to a particular bacterial species or type. When compared with probes derived from whole genomic libraries, probes to rRNA, especially those complementary to hypervariable regions of rRNA, are advantageously sensitive and specific in their ability to detect low numbers of bacteria and to distinguish between bacterial species, types, and subtypes. It is preferred that the assays comprise capture oligonucleotides to two different hypervariable regions of the same bacterial ribosomal

RNA. Such a format dramatically reduces the incidence of false negative results due to heterogeneity in the selected target regions. The methods disclosed herein have the advantage of being rapid assays. The disclosed methods are relatively simple to conduct, and they have a degree of reproducibility and accuracy that has heretofore not been achieved for bacterial pathogens of the mouth.

#### Sample Collection

Microbial specimens for use in this invention can be obtained from the ATCC for any of the bacteria listed in Table 1. For clinical studies, samples are obtained from teeth scrapings of subgingival plaque or from fluid taken from periodontal pockets with paper points of human patients suspected of having periodontal disease. The samples are dispersed in a buffer, which provides a biologically compatible solution, such as 150mM NaCl, 20mM Tris-HCl (pH 7.5), 10mM EDTA, 10mM EGTA, or 150mM NaCl, 20mM  $\text{NaPO}_4$  (pH 7.5), 10mM EDTA, 10mM EGTA, and frozen until use. Prior to assay, the cells are optionally cultured in accordance with standard microbiological techniques, such as those described by Dzink, et al., J. Clin. Micro., 19:599, 1984. The bacteria are lysed in a lysis buffer containing N-acetylmuramidase (lysozyme). Lysing buffers are known in the art. EP 199,439; Potts, T.V. and Berry, Em. Internat. J. Sys. Bacter., 33:765-771 (1983); Bonta, Y., et al., J. Dent. Res., 64:793-798 (1985). Generally, these buffers are between pH 7.0 and 8.0, containing both chelating agents and surfactants. Heat denaturing is optional.

Alternatively, samples are dispersed and collected directly in a lysing solution that also functions as a hybridization solution, such as 3M guanidinium thiocyanate (GuSCN), 50mM Tris (pH 7.6), 10mM EDTA, 0.1% sodium dodecylsulfate (SDS), and 1% mercaptoethanol (Maniatis, T. et al. Molecular Cloning:

A Laboratory Manual, Cold Spring Harbor, New York, 1982).  
Lysis is performed at 65°C for 10 min.

Probes Complementary to the rRNA of Orally Pathogenic Bacteria

5           This invention is directed to probes complementary to hypervariable regions of the rRNA sequence that are not conserved between bacterial species or types, and these probes can thus distinguish between species or types. Five sequences within 16S rRNA were  
10 derived using three universal oligonucleotide primers described by Lane et al., Proc. Natl. Acad. Sci., U.S.A., 82:6955-6959 (1985), and the following three oligonucleotide primers developed by us:

UP4B: 5'GCTGGCACGGAGTTAGCCG3';  
15           UP8B: 5'CACGARCTGACGACARCCATGC3'; and  
UP6B: 5'TACGGNTACCTTGTTACGAC.

The nomenclature for incompletely specified bases in the nucleic acid sequences are those recommended by the International Union of Biochemistry, Proc. Natl. Acad.  
20 Sci., U.S.A. 83:4-8 (1986), which is herein incorporated by reference. For example, nucleotides designated R are interchangeable with guanosine, adenosine, or their analogs; nucleotides designated N means any of the natural nucleosides or their analogs may be interchanged  
25 at this position. Nucleotides designated Y are interchangeable with cytosine or thymine, nucleotides designated W are interchangeable with adenosine or thymine; nucleotides designated M are interchangeable with adenosine or cytosine.

30           These oligonucleotide primers are specific for conserved regions of the prokaryote 16S rRNA, and can be used to prime whole nucleic acid preparations without pretreatment with DNase or physical separations of RNA from DNA. Commercial preparations of reverse  
35 transcriptase, along with appropriate primer extension reactants for sequencing on electrophoretic gels, are



used to obtain transcripts of cDNA complementary to the hypervariable regions of each pathogenic bacteria. The preferred sequencing methodology will use dideoxynucleotide chain termination. Similarly, oligonucleotide primers specific for conserved regions of the prokaryote 23S rRNA (Table 2) have been used to sequence nucleic acid preparations using known sequencing methodology.

The oligonucleotide probes shown in Table 2 are complementary to conserved regions of the prokaryote 16S and 23S ribosomal RNA, and will hybridize under hybridizing conditions to ribosomal RNA and its corresponding genomic DNA sequence. These universal oligonucleotide probes are also ideally suited to be utilized as signal oligonucleotides to DNA and especially RNA of all bacteria. In the multiple dipstick format (see later) the target nucleic acids are captured by probes specific for hypervariable regions while only one universal signal probe derived from the conserved regions of ribosomal RNA can be used to detect the nucleic acid from all the different pathogenic bacteria. In addition, it is possible to increase the strength of the signal by using more than one universal signal oligonucleotide probe.

After sequence analysis of the pathogens is complete for the 16S or 23S rRNA, the sequences can be compared by commercially available computer programs, such as the MicroGenie Program, sold by Beckman Instruments (Palo Alto, California). Present programs are limited in that only two sequences can be compared at a time and all nucleotides are given equal importance. A more complex comparison can be made, but a more powerful computer (e.g., a Cray computer) is required. Waterman, M.S. Multiple Sequence Alignment by Consensus. Nuc. Acids. Res. 14 (1986), 9095-9102. The preferred method for selection requires alignment of multiple sequences and the identification of conserved blocks. Probes are then chosen to include maximum diversity (e.g.,



insertions or deletions instead of transitions). The probes may be either DNA or RNA, although DNA probes are preferred because they can be chemically synthesized.

The degree of complementarity (homology) required for detectable binding with the rRNA of pathogenic bacteria will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100 percent; however, it should be understood that minor variations in the rRNA may be compensated for by reducing the stringency of the hybridization and/or wash medium as described below. Thus, despite the lack of 100 percent complementarity under reduced conditions of stringency, functional probes having minor base differences from their rRNA targets are possible. Therefore, under hybridization conditions of reduced stringency, it may be possible to modify up to 60% of a given oligonucleotide probe while maintaining an acceptable degree of specificity. In addition, analogs of nucleosides may be substituted within the probe for naturally occurring nucleosides. This invention is intended to embrace these species when referring to polynucleic acid probes.

To obtain large quantities of DNA or RNA probes, one can either clone the desired sequence using traditional cloning methods, such as described in Maniatis, T., et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1982, or one can produce the probes by chemical synthesis using commercially available DNA synthesizers. An example of cloning would involve insertion of the cDNA for the ribosomal RNA into a replication vector, such as pBR322, M13, or into a vector containing the SP6 promoter (e.g., generation of single-stranded RNA using SP6 RNA polymerase), and transformation of a bacterial host. The DNA probes can be purified from the host cell by lysis and nucleic acid extraction, treatment with selected restriction enzymes, and further isolation by gel

electrophoresis.

DNA probes may be chemically synthesized using commercially available methods and equipment. For example, the solid phase phosphoramidite method can be used to produce short probes of between 15 and 50 bases. For this invention, it is preferred to chemically synthesize short DNA probes using the Model 380B DNA Synthesizer from Applied Biosystems, Foster City, California, using reagents supplied by the same company.

Probes may be comprised of the natural nucleotide bases or known analogs of the natural nucleotide bases, including those modified to bind labeling moieties.

The oligonucleotide sequences in Table 1 are representative of probes that hybridize with and detect specific regions of specific 16S rRNAs. The oligonucleotide sequences in Table 3 are representative of probes that hybridize with and detect hypervariable regions of specific 23S rRNAs, and should not be viewed as limiting. Probes may consist of these sequences or equivalent sequences, by themselves as a single unit for binding, or may be comprised of additional sequences not having the capacity to bind to non-pathogenic associated bacteria. Probes comprising more than the short sequences, as offered in Table 1, may have repeating units of the same sequence (e.g., concatemers of a sequence), a mixture of different sequences specific to one species of bacteria, and even a mixture of sequences that may be specific to one or more bacterial species associated with oral diseases.

If such probes are to contain concatemers of short sequences, said long probes will display the high hybridization specificity inherent in a "short" probe containing, for example, only 20 nucleotides. This concatemeric probe sequence could be contained within the cloning vector sequences and would have the structure given by the formula below. Alternatively, the concatemer having the formula  $[X-Y-Z]_n$  as previously defined

could be excised from the cloning vector with the appropriate restriction endonuclease.

Probes may be labeled by any one of several methods typically used to detect the presence of hybrid polynucleotides. The most common method of detection is the use of autoradiography with  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$  labeled probes or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability and half lives of the selected isotopes. Other labels include ligands which bind to antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. Alternatively, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The choice of label dictates the manner in which the label is bound to the probe. Radioactive probes are typically made using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes, for example, by using DNA synthesizers, by nick translation with DNA polymerase I, by tailing radioactive DNA bases to the 3' end of probes with terminal deoxynucleotidyl transferase, by treating single-stranded M13 plasmids having specific inserts with the Klenow fragment of DNA polymerase in the presence of radioactive deoxynucleotides, dNTP, by transcribing from RNA templates using reverse transcriptase in the presence of radioactive deoxynucleotides, dNTP, or by transcribing RNA from vectors containing specific RNA viral promoters (e.g., SP6 promoter) using the corresponding RNA polymerase (e.g., SP6 RNA polymerase) in the presence of radioactive ribonucleotides rNTP.

The probes can be labeled using radioactive nucleotides in which the isotope resides as a part of the

nucleotide molecule, or in which the radioactive component is attached to the nucleotide via a terminal hydroxyl group that has been esterified to a radioactive component such as inorganic acids, e.g.,  $^{32}\text{P}$  phosphate or  $^{14}\text{C}$  organic acids, or esterified to provide a linking group to the label. Base analogs having nucleophilic linking groups, such as primary amino groups, can also be linked to a label.

Non-radioactive probes are often labeled by indirect means. For example, a ligand molecule is covalently bound to the probe. The ligand then binds to an anti-ligand molecule which is either inherently detectable or covalently bound to a detectable signal system, such as an enzyme, a fluorophore, or a chemiluminescent compound. Ligands and anti-ligands may be varied widely. Where a ligand has a natural anti-ligand, namely ligands such as biotin, thyroxine, and cortisol, it can be used in conjunction with its labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

Probes can also be labeled by direct conjugation with a label. For example, cloned DNA probes have been coupled directly to horseradish peroxidase or alkaline phosphatase, (Renz, M., and Kurz, K. A Colorimetric Method for DNA Hybridization. Nuc. Acids Res. 12:3435-3444, 1984) and synthetic oligonucleotides have been coupled directly with alkaline phosphatase (Jablonski, E., et al., Preparation of Oligodeoxynucleotide-Alkaline Phosphatase Conjugates and Their Use as Hybridization Probes. Nuc. Acids. Res. 14:6115-6128, 1986, and Li P., et al., Enzyme-linked Synthetic Oligonucleotide probes: Non-Radioactive Detection of Enterotoxigenic Escherichia Coli in Faeca Specimens. Nuc. Acids Res. 15:5275-5287 (1987).

Enzymes of interest as labels will primarily be hydrolases, such as phosphatases, esterases and glyco-

sidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescers include luciferin, and  
5 2,3-dihydrophthalazinediones, e.g., luminol.

#### Hybridization Conditions

Various hybridization solutions may be employed, comprising from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization  
10 solution employs about 50% v/v formamide, about 0.5 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9), about 0.05 to 0.2% detergent, such as sodium dodecylsulfate, or between 0.5-20mM EDTA, ficoll (about  
15 300-500 kilodaltons), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/ml, fragmented nucleic DNA, e.g., calf thymus or salmon sperm DNA, or  
20 yeast RNA, and optionally from about 0.5 to 2% wt./vol. glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as polyethylene glycol, anionic polymers such as polyacrylate or poly-  
25 methylacrylate, and anionic saccharidic polymers, such as dextran sulfate.

An alternative hybridization solution may be employed comprising about 2 to 4M GuSCN, preferably 3M, about 0.01 to 0.1M Tris (pH range about 6.0 to 8.5), a  
30 detergent such as sodium dodecyl sulfate in concentrations of about 0.1 to 5% (w/v), and about 0.01 to 0.1M EDTA. Other additives may also be included such as carrier DNA or RNA, or protein such as bovine serum albumin or gelatin. Stringency of the hybridization  
35 solution can be adjusted by the addition of about 0 to 10% formamide, usually 5%.



The particular hybridization technique is not essential to the invention. Hybridization techniques are generally described in Nucleic Acid Hybridization: A Practical Approach, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1987; Gall and Pardue (1969), Proc. Natl. Acad. Sci., U.S.A., 63:378-383, and John, Burnsteil and Jones (1969) Nature, 223:582-587. As improvements are made in hybridization techniques, they can readily be applied. One such improvement is the subject of Serial No. 130,754, our docket 11652-5, filed on December 9, 1987, incorporated herein by reference, which relates to the use of ultrasonic energy to enhance the rate of hybridization. This is an optional step which does not influence the specificity of the probes described herein.

The amount of labeled probe which is present in the hybridization solution may vary widely, depending upon the nature of the label, the amount of the labeled probe which can reasonably bind to the cellular target nucleic acid, and the stringency of the hybridization medium and/or wash medium. Generally, substantial excesses of probe over the stoichiometric amount of the target nucleic acid will be employed to enhance the rate of binding of the probe to the target DNA.

Various degrees of stringency of hybridization can be employed. As the conditions for hybridization become more severe, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. The degree of stringency can be controlled by temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of formamide within the range of 0% to 50%. Temperatures employed will normally be in the range of about 20° to 80°C, usually 30° to 75°C. For probes of 15-50 nucleotides in 50% formamide, the optimal temper-



ature range can vary from 22° to 65°C. With routine experimentation, one can typically define conditions which permit satisfactory hybridization at room temperature.

5. Assay test protocols for use in this invention are those of convention in the field of nucleic acid hybridization, and include both single phase, where the target and probe polynucleic acids are both in solution, and mixed phase hybridizations, where either the target or probe polynucleotides are fixed to an immobile support. The assay test protocols are varied and are not to be considered a limitation of this invention. A general review of single phase hybridization can be had from a reading of Nucleic Acid Hybridization: A Practical Approach, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1985, and Hybridization of Nucleic Acids Immobilized on Solid Supports, Meinkoth, J. and Wah, G., Analytical Biochemistry, pp. 238, 267-284, 1984. Mixed phase hybridizations are preferred.

20 Cultured colonies of the bacteria can be assayed using colony hybridization techniques, wherein the bacteria suspected of being orally pathogenic are plated and adsorbed onto a filter prior to exposure to the target polynucleotides (Grunstein and Hogness, Colony Hybridization in Methods of Enzymology, Ed. Ray Wu, 1979, Vol. 68, pp. 379-409, and Proc. Natl. Acad. Sci. U.S.A., Vol. 72, No. 10:3961-3965, 1975). The colonies may also be identified by electrophoretically separating the nucleic acid and exposing the separated species to those probes designed to distinguish between species of pathogenic oral bacteria. Ribosomal RNA may be detected using the methods of Alwine, J.C., et al., Proc. Natl. Acad. Sci. U.S.A., Vol. 74(12): 5350-5354 (1977). Techniques for the electrophoretic separation of DNA, including ribosomal genomic regions, are described in WO 35 83/01073.

Methods for in situ hybridization are also applicable to this invention. In situ hybridization refers to the identification of bacterial cells using polynucleotide probes, wherein the intact cell is immobilized and provided as a target. The following two review articles provide an overview of the art of in situ hybridization: Singer, R.H., et al., Biotechniques, 4(3):230-250 (1986), and Haase, Al, et al., Methods in Virology, Vol. VII, pp. 189-226 (1984), and are incorporated by reference herein.

Nucleic acids from GuSCN-lysed bacteria can be immobilized directly on to nitrocellulose or Nytran, and hybridized with the appropriate probe. The GuSCN-lysate is diluted with buffer containing formaldehyde, slotted to nitrocellulose and heated at 80°C to denature the nucleic acids.

Regardless of the assay test protocol being used, the bacterial cells are to remain in contact with a hybridization solution at a moderate temperature for an extended period of time. In single phase assays, the double-stranded duplexes may be separated from single-stranded nucleic acid by  $S_1$  nuclease digestion followed by precipitation of duplex molecules, or by selective binding to hydroxyapatite. In mixed phase assays, the support-immobilized nucleic acids is introduced into a wash solution having analogous concentrations of sodium chloride, buffers, and detergent, as provided in the hybridization solution. The time period for which the support is maintained in the wash solution may vary from several minutes to three hours or more.

Either the hybridization or the wash medium can be stringent. Typically, for mixed phase assays, it is the wash solution that most often determines the stringency and facilitates dissociation of mismatched duplexes. After rinsing the support at room temperature with a dilute buffered sodium chloride solution, the support may now be assayed for the presence of duplexes

in accordance with the nature of the label.

Where the label is radioactive, the presence of probe can be detected in a scintillation counter. More conveniently, in mixed phase assays, the substrate can be  
5 dried and exposed to X-ray film in any number of conventional autoradiographic protocols.

Where the label is fluorescent, the sample is detected by first irradiating it with light of a particular wavelength. The sample absorbs this light and then  
10 emits light of a different wavelength which is picked up by a detector (Physical Biochemistry, Freifelder, D., W.H. Freeman & Co., pp. 537-542, 1982).

Where the label is a hapten or antigen, the sample can be detected by using antibodies. In these  
15 systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies; in some cases the antibody is labeled with a radioactive probe. (Tijssen, P., Practice and Theory of Enzyme Immunoassays, Laboratory Techniques in Biochemistry and Molecular  
20 Biology, Burdon, R.H., van Knippenberg, Ph.H., Eds., Elsevier, pp. 9-20, 1985.)

One method of detection is enzymatic detection in conjunction with biotin. Although fluorescence is an alternative label, enzymatic labels, in combination with  
25 avidin or streptavidin such as biotinylated peroxidase or alkaline phosphatase, are preferred. Enzyme-conjugated avidin or streptavidin can also be used to directly bind the enzyme to the probe (Haase, et al., supra). Preferred enzymes are peroxidase or alkaline phosphatase.  
30 An especially preferred method utilizes enzymes directly conjugated to probes. The preferred enzymes are alkaline phosphatase and peroxidase. Methods for conjugating enzymes to oligonucleotides are known. Nucleic Acid Res., 14:6115-6128 (1986) and Nucl. Acid Res., 15:5275-  
35 5287 (1987).

The preferred assay protocol involves direct testing of samples without culturing. These protocols

demand additional sensitivity for which the invention has particular application. Samples suspected of containing oral pathogenic bacteria are first subjected to a lysing solution, such as a buffered solution of detergent and a  
5 divalent metal chelator or a buffered chaotropic salt solution containing a detergent, a reducing agent and a divalent metal chelator. The sample may be directly fixed to a support or further processed to extract nucleic acids. Released or extracted bacterial nucleic  
10 acid (including target nucleic acid) are fixed to a solid support, such as cellulose, nylon, nitrocellulose, diazobenzyloxymethyl cellulose, and the like.

In the preferred instance, the assay protocol is a sandwich-type assay. A primary component of a  
15 sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the rRNA sequence. Preferred are those probes that hybridize to regions of  
20 the ribosomal RNA with minimal secondary and tertiary interactions, such as those listed in Table 4. The advantage of such probes is that the hybridization can be carried out without the additional step of heat denaturing the sample nucleic acid. The test sample  
25 suspected of containing oral pathogenic bacteria is then contacted with the solid support in a hybridization medium. Finally, a second soluble-labeled probe complementary to a different sequence of the rRNA of the pathogenic bacteria is hybridized to the rRNA that has  
30 formed a hybridization duplex with the immobilized nucleic acid probe on the solid support.

The presence of the bacteria is then determined in accordance with the label being used. It should be noted that the second probe can be added simultaneously  
35 with the test sample to the hybridization assay. In addition the second probe can hybridize to either a conserved or to a hypervariable region of the rRNA.

Preferred are the probes derived from conserved regions of the ribosomal RNA with minimal secondary and tertiary interactions (Table 4), such as UP7B or UP9A for 16S ribosomal RNA, and UP12B or 23UPB for 23S ribosomal RNA.

5 The advantage of such probes is that the hybridization can be carried out without the additional step of heat denaturing the nucleic acid. A general reference for various detection methods can be found in Hames, B.D. and Higgins, S.J., Nucleic Acid Hybridization, IRL Press, 10 Oxford, 1985. References for sandwich assay with DNA probes are Dunn and Hassell Cell, Vol. 12, pp. 23-26, 1977, and Ranki, et al., U.S. Patent No. 4,486,539.

The oligonucleotide or polynucleotide acid probes of this invention can be included in a kit which can be

15 used to rapidly determine the presence or absence of oral pathogenic bacteria, especially the species disclosed in Table 1. The kit includes all components necessary to assay for the presence of these bacteria. In the universal concept, the kit includes a stable preparation

20 of labeled probes to rRNA, hybridization solution in either dry or liquid form for the hybridization of target and probe polynucleotides, as well as a solution for washing and removing undesirable and nonduplexed polynucleotides, a substrate for detecting the labeled duplex, and optionally an instrument for the detection of

25 the label.

A more specific embodiment of this invention embraces a kit that utilizes the concept of the sandwich assay. This kit would include a first component for the

30 collection of samples from the mouths of patients, such as a scraping device or paper points, vials for containment, and buffers for the dispersement and lysis of the sample. A second component would include media in either dry or liquid form for the hybridization of target

35 and probe polynucleotides, as well as for the removal of undesirable and nonduplexed forms by washing. A third component includes a solid support upon which is fixed or



to which is conjugated unlabeled nucleic acid probe(s) that is(are) complementary to a part of either the 16S or 23S rRNA of the species of bacteria being tested. In the case of multiple target analysis more than one capture probe, each specific for its own ribosomal RNA, will be applied to different discrete regions of the dipstick. A fourth component would contain labeled probe that is complementary to a second and different region of the same rRNA strand to which the immobilized, unlabeled nucleic acid probe of the third component is hybridized. The probe components described herein include combinations of probes in dry form, such as lyophilized nucleic acid or in precipitated form, such as alcohol precipitated nucleic acid or in buffered solutions. The label may be any of the labels described above. For example, the probe can be biotinylated using conventional means and the presence of a biotinylated probe can be detected by adding avidin conjugated to an enzyme, such as horseradish peroxidase, which can then be contacted with a substrate which, when reacted with peroxidase, can be monitored visually or by instrumentation using by a colorimeter or spectrophotometer. This labeling method and other enzyme-type labels have the advantage of being economical, highly sensitive, and relatively safe compared to radioactive labeling methods. The various reagents for the detection of labeled probes and other miscellaneous materials for the kit, such as instructions, positive and negative controls, and containers for conducting, mixing, and reacting the various components, would complete the assay kit.

The following examples are offered by way of illustration and not by limitation.

#### Example 1

##### A. Preparation of rRNA

Bacterial cells are resuspended in a lysis solution (20 mg/ml lysozyme, 25% sucrose, 50mM Tris, pH



8, 10mM EDTA), and incubated at 37°C for 30 min. Sodium dodecylsulfate (1-2% w/v) and pronase E (1 mg/ml) or proteinase K (200 µg/ml) are added, and the solution is incubated 30 min at 37°C. The lysates are extracted twice with phenol:chloroform (1:1, v/v) and then precipitated with ethanol. Nucleic acid is pelleted, washed with 70% v/v ethanol, and resuspended to approximately 1 mg/ml in 1X TE buffer (10mM Tris, pH 8, 1mM EDTA). Resuspended nucleic acid is stored at -70°C.

10 B. Oligonucleotide Sequencing Primers

Universal sequencing primers (UP) for rRNA were as described by Lane, et al., Proc. Natl. Acad. Sci. U.S.A., 82, 6955-6959 (1985). Three additional primers, herein designated UP4B, UP8B and UP6B, and having the sequences

UP4B: GCTGGCACGGAGTTAGCCG;

UP8B: CACGARCTGACGACARCCATGC;

UP6B: TACGGNTACCTTGTTACGAC;

were designed and utilized for sequencing.

20 C. Sequencing Reactions

The protocol for sequencing is a modification of the dideoxynucleotide-terminated chain elongated method described by Sanger, F., et al., Proc. Natl. Acad. Sci. U.S.A., 74, 5463-5467 (1977), as adapted for rRNA templates by Lane, et al. Proc. Natl. Acad. Sci. U.S.A., 82, 6955-6959 (1985). Sequencing primers were radioactively labeled with 32P-ATP using T4 polynucleotide kinase (50uCi 32P-ATP, 3000Ci/mMol, per 300 ng oligo and 10 units of kinase). The radioactively labeled primers were ethanol precipitated and resuspended in water to 30ng/ml.

A standard sequencing reaction is set up as follows. Two µl of primer are added to 3 µl of a solution of bacterial nucleic acid (0.5-25 mg/ml), 2 µl 5X HYB buffer (500mM KCl, 250mM Tris-HCl, pH 8.5), and 3 µl

of H<sub>2</sub>O. The mix is heated at 100°C for 2 min, then cooled to room temperature. To this mix is added 4 µl of 5X RT buffer (250mM Tris-HCl, pH 8.3, 250mM KCl, 50mM dithiothreitol, 50mM MgCl<sub>2</sub>), 5 µl H<sub>2</sub>O, and 2 µl reverse transcriptase (200 U/µl, Bethesda Research Laboratories, Gaithersburg, Maryland). Three µl aliquots are pipetted into 2 µl deoxynucleotide/dideoxynucleotide mixes (A: 17 µM dATP, 300 µM dCTP, 330 µM dGTP, 330 µM TTP, 125 µM ddATP; C: 330 µM dATP, 17 µM dCTP, 330µm dGTP, 330 µM TTP, 125 µM ddCTP; G: 330 µM dATP, 330 µM dCTP, 17 µM dGTP, 330 µM TTP, 250 µM ddGTP; T: 330 µM dATP, 330 µM dCTP, 330 µM dGTP, 17 µM TTP, 125 µM ddTTP). The reactions are incubated at 43°C for 15 min. Two µl of chase (330 µM dATP, dCTP, dGTP, TTP, plus 400 units of reverse transcriptase) are added to each reaction. The reactions are incubated for an additional 15 min at 43°C, and terminated by the addition of 6 µl loading solution (95% formamide, 2.5% xylene cyanol, 2.5% bromphenol blue). Reaction products were electrophoresed on 8% polyacrylamide denaturing gels and visualized by autoradiography. The sequencing reactions can also be performed using unlabeled sequencing primer and [<sup>35</sup>S]dATP under reaction conditions similar to those described above.

25 D. Synthesis and Purification of Synthetic Oligonucleotide Probes

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer Model 380B via B-cyanoethyl-phosphoramidite chemistry. The oligonucleotides were purified by preparative polyacrylamide gel electrophoresis or by high pressure liquid chromatography and eluted as detailed in Applied Biosystems User Bulletin No. 13: "Evaluation and Purification of Synthetic Oligonucleotides," November 9, 1984. See Table 1 for preferred sequences.

E. Isolation of Nucleic Acid from Bacterial Culture or Subgingival Plaque Samples

The following procedure is applicable to either a curette scraping or cultured bacteria: 500  $\mu$ l of sample (stored at  $-70^{\circ}\text{C}$ ) is thawed with the following steps to be conducted immediately. 100 ng of an unrelated carrier oligonucleotide (24 bases) is added to the sample if it is a plaque sample or a dilute bacterial sample. 250  $\mu$ l of sucrose lysis buffer (75% (w/v) sterile sucrose, 10mM EDTA, 10mM EGTA, 50mM Tris-HCl (pH 8.0), is added to the sample and vortexed briefly. 50  $\mu$ l of freshly made lysozyme (10 mg/ml in 0.25X bacterial sucrose lysis buffer; Sigma Chemical), is added and the sample incubated for 15 min at  $37^{\circ}\text{C}$ . 75  $\mu$ l of 10% SDS is then added and the sample vortexed briefly. 75  $\mu$ l of Pronase E (10 mg/ml, Sigma Chemical; self-digested as per Maniatis, et al., Molecular Cloning: A Laboratory Manual) is added, the sample vortexed briefly and incubated at  $37^{\circ}\text{C}$  for 30 min. The sample is extracted two times with phenol:chloroform:isoamyl alcohol (24:24:1, v/v/v), saving the aqueous phase. If the interphase is not clear, it can be extracted again with chloroform and the aqueous phase saved. The sample is then concentrated by lyophilization to 300  $\mu$ l, to which 150  $\mu$ l of a sterile 7.5M  $\text{NH}_4\text{OAc}$  solution is added, and followed by 1.12 ml of 95% ethanol. The sample is then mixed and stored at  $-70^{\circ}\text{C}$  for 30 min or longer to precipitate the nucleic acid, and the pellet is recovered by centrifugation for 10 min at  $4^{\circ}\text{C}$ . The nucleic acid pellet is washed once with 1.5 ml of 95% ethanol, dried briefly, and then resuspended in 400  $\mu$ l of sterile TE buffer (10mM Tris-HCl pH 7.5, 0.1mM EDTA).

F.a) Immobilization of Nucleic Acid onto Nytran Membranes

35 The extracted microbial nucleic acids are immobilized onto Nytran as follows: 380  $\mu$ l of nucleic

acid (at less than 6  $\mu\text{g}/380\ \mu\text{l}$ ) in TE and 20  $\mu\text{l}$  of 200mM Pipes (pH 7.42) are mixed to give a final concentration of 20mM Pipes. The sample is heated to 100°C for 90 sec, quickly chilled in a ice/water bath, and then applied to  
5 a Nytran membrane using a slot blot apparatus from Schleicher and Schuell (Keene, New Hampshire). Slotting should be finished within 20 min of the heat treatment. The slots are washed with 200  $\mu\text{l}$  of 10X SSC (1.5M NaCl, 0.15M sodium citrate pH 7.0), the Nytran membrane with  
10 immobilized nucleic acid baked at 80°C for 1 hour, and the Nytran membrane stored at room temperature between blotting paper.

F.b) Direct immobilization of cell lysates to nitrocellulose membranes

15 A cell pellet of cultured bacteria or a plaque sample is resuspended in 100ul of 3M GuSCN, 2% sarkosyl w/v, 50mM Tris-HCl (pH 7.6), 10mM EDTA, and 1% v/v mercaptoethanol and incubated at room temperature for 10 min. To this solution is added 0.3 volumes of 20X SSC,  
20 followed by 0.2 volumes of 37% formaldehyde, and incubation at 55°C for 15 min. Serial dilutions are made in 15X SSC and the nucleic acid solutions slotted onto nitrocellulose (prewetted with water and soaked in 6X SSC for 10 min). The filters are baked at 80°C under  
25 vacuum for 2 hours and stored at room temperature between blotting paper.

G. Hybridization

Procedure A

Probes are labeled with  $^{32}\text{P}$  by polynucleotide  
30 kinase as per Maniatis, T. et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1982, (200 ng of oligonucleotide, 60  $\mu\text{Ci}$   $^{32}\text{P}$ -ATP, and 40 units of  $T_4$  polynucleotide kinase in a 25  $\mu\text{l}$  volume) and purified by ethanol precipitation and Elutip-D  
35 chromatography (Scheicher and Schuell). Alternatively,

probes are synthesized with an ethylamine group at the 5' end, biotinylated with NHS-LC-biotin (Pierce Chemical Co., Rockford, Illinois), and purified by Elutip-D chromatography. Nucleic acids, immobilized on Nytran or nitrocellulose membranes, are hybridized with 5 ng/ml of oligonucleotide probe in 0.6M NaCl, 90mM Tris-HCl (pH 8.0), 10mM EDTA, 5X Denhardt's solution, 30% deionized formamide, 0.1% SDS, and 100  $\mu$ l/ml fragmented yeast RNA at 42°C, or in 0.9M NaCl, 90mM Tris-HCl (pH 8.0), 10mM EDTA, 5X Denhardt's solution, 0.1% SDS, and 100  $\mu$ g/ml fragmented yeast RNA at 50°C. The hybridizations are carried out in 50 ml polypropylene tubes or with sealed "Micro-Seal" plastic bags (Dazey Corp., Industrial Airport, Kansas) on a rotary shaker at the appropriate temperature for 1 hour or longer. The membranes are first washed in 0.09M NaCl, 9mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.1% SDS at room temperature, and then washed in the same wash buffer at 50°C (=stringent wash). An alternate stringent wash can be conducted with 3.1M tetraethylammonium bromide, 50mM Tris-HCl (pH 8.0), 2mM EDTA, and 0.1% SDS at 29°C (this procedure requires a 0.9M NaCl, 90mM Tris-HCl (pH 8.0), 10mM EDTA pre-wash prior to the tetraethylammonium bromide wash).

#### Procedure B

Nucleic acids immobilized on membranes are hybridized with 5ng/ml oligonucleotide probe in 3M GuSCN, 5% formamide, 50mM Tris(pH 7.6), 10mM EDTA, 2% sarkosyl for 0.5 to 24 hours as described in procedure A. The membrane is washed twice for 2 min with a solution containing 0.09M NaCl, 9mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.1% SDS at room temperature. When  $^{32}$ P labeled probes were used, nucleic acids were visualized by autoradiography. The use of biotinylated probes required the incubation with streptavidine-alkaline phosphatase or streptavidine-peroxidase followed by color development as described in Example 2.



#### H. Detection of Hybridized Probe

Hybridized probe labelled with  $^{32}\text{P}$  was detected by autoradiography. Biotinylated probes were detected by the Detek-alk kit (avidin-alkaline phosphatase; Enzo Biochem, New York, New York) or the DNA detection kit (streptavidin/biotinylated alkaline phosphatase polymer; Bethesda Research Labs, Gaithersburg, Maryland).

#### I. Identification of Synthetic Oligonucleotide Probes Complementary to regions with minimal secondary and tertiary interaction

The chemical and enzymatic accessibility of nucleotides along the predicted secondary structure of the 16S and 23S ribosomal RNA have been observed. Moazed, D. et al. (1986) J. Mol. Biol. 187:399-416. Noller, H.F. et al. (1981) Nucleic Acids Res. 9:6167-6189. However, the accessibility of these regions to complementary oligonucleotide probes is not predictable. Lasater, L.S. et al. (1988) Biochemistry 28:4687-4695. Regions of the ribosomal RNA with minimal secondary and tertiary interactions are defined by solution hybridization and sandwich assay methods. For example, purified ribosomal RNA (1-5 ug) was hybridized with  $^{32}\text{P}$ -labelled complementary oligonucleotide probes (5-10 ng), such as those in Table 1 or Table 2, in 0.09M NaCl, 9mM Tris-HCl (pH 8.0), and 1mM EDTA, or 3M GuSCN, 2% sarkosyl, 50mM Tris-HCl (pH 7.6), 10mM EDTA, 1% mercaptoethanol, and 5% formamide.

Following hybridization for 15 min to 1 hour, the sample was subjected to agarose gel electrophoresis to separate the 16S and 23S ribosomal RNA subunits, and the oligonucleotide probe:rRNA hybrids visualized by autoradiography. Oligonucleotide probes which showed hybridization specific to their respective ribosomal RNA subunit were further tested in the sandwich assay format. In this format the test oligonucleotide probe was covalently attached to a solid support. Target ribosomal



RNA was hybridized in the presence of the test oligonucleotide and  $^{32}\text{P}$ -labelled signal oligonucleotide probes, such as UP7B or UP9A for 16S ribosomal RNA, or UP12B or 23UPB for 23S ribosomal RNA, using the GuSCN hybridization solution described above in Procedure B. After hybridization for 2-15 hours, the solid support was washed of unhybridized nucleic acid, and the amount of ribosomal RNA captured by the test oligonucleotide probe quantitated by liquid scintillation counting.

#### 10 Example 2

The direct conjugation of alkaline phosphatase to the oligonucleotide probes will eliminate several steps required in the assay procedures using oligonucleotide probes (e.g., biotinylated probes) which are indirectly linked with enzymes.

Direct conjugation of alkaline phosphatase to a short probe involves conjugation via heterobifunctional reagents. The synthetic probe is synthesized as described herein with a linker arm reagent attached to the terminal 5'-hydroxyl.

An aminohexyl linker arm with a terminal amino group is attached to the 5'-hydroxyl of the synthetic oligodeoxynucleotide as the last step in its synthesis on an automated DNA synthesizer. The reagent used for linker arm introduction is 6-(methoxytritylamino)hexyl 2-cyanoethyl *N,N*-diisopropylphosphoramidite, prepared from 6-aminohexanol in a manner similar to the synthesis of the 3-(methoxytritylamino)propyl methyl *N,N*-diisopropylphosphoramidite, as described by B.A. Connolly, Nucl. Acids Res., 15:3131 (1987). The linker arm is attached to the probe, and the deprotected probe purified in a method similar to the methods described in the Connolly reference.

The oligonucleotide first is derivatized with the thiol-reactive agent *N*-succinimidyl(4-iodoacetyl)aminobenzoate ("SIAB") through the amino linker arm. The

SIAB-oligonucleotide is prepared by adding 1.2 mg SIAB to 300  $\mu$ g of the oligonucleotide, incubating for one hour at room temperature, and desalting over a G-25 column equilibrated with 20mM sodium phosphate (pH 6.0), 5mM EDTA. This probe derivative can be coupled to alkaline phosphatase which is modified as described below.

Alkaline phosphatase is thiolated with dithio-bis(succinimidylpropionate) ("DSP") by adding 800  $\mu$ g DSP to 4 mg alkaline phosphatase. The reaction is allowed to proceed for 30 min at room temperature. The reaction mixture then is treated with dithiothreitol for 15 min at room temperature to reduce the disulfide, and desalted over a G-25 column equilibrated with 20mM sodium phosphate (pH 6.0).

The SIAB-oligonucleotide is mixed with the DSP-alkaline phosphatase at a oligonucleotide:alkaline phosphatase ratio of 4:1. 5M NaCl is added to the reaction to bring the final NaCl concentration to 3M, and the pH is adjusted to 7.5 with 0.1 volume 1M Tris (pH 7.5). The coupling reaction is allowed to proceed overnight (16 hours), then stopped with N-ethylmaleimide (2  $\mu$ l of 10ng/ml). The conjugate is separated from free oligonucleotide by gel filtration on a P-100 column, and free alkaline phosphatase is removed by DEAE cellulose chromatography.

Horseradish peroxidase (HRP) is first treated with  $\text{NaIO}_4$  ( $\text{IO}_4$ :HRP, 160:1) to oxidize the sugar diols. After 15 min at room temperature the  $\text{IO}_4$  is removed by gel filtration over Sephadex G-25, and the material concentrated to less than 20 mg/ml in 1mM NaOAc (pH 4.5). The concentrated HRP is then used to resuspend a pellet of oligonucleotide with a 5'-hexylaminelinker arm. Following resuspension, the pH of the reaction mix is adjusted to 9.5 with carbonate buffer and  $\text{NaBH}_3\text{CN}$  is added to 50mM. The reaction is allowed to proceed for 16 to 20 hours at room temperature and the products separated by HPLC using a GF-250 column (Dupont). The

probe-enzyme conjugates are tested in the following ways. *Fusobacterium nucleatum* or *Bacteroides gingivalis* genomic DNA was serially diluted into human placental DNA, heat denatured, and applied to slots on a Nytran membrane.

5 Bg-1B:HRP (100 ng/ml in 30% formamide, 0.6M NaCl, 90mM Tris-HCl (pH 8.0), 10mM EDTA, and 0.1% SDS) was hybridized to the immobilized DNA for 1 hour at room temperature, and washed in 0.45M NaCl, 45mM Tris-HCl pH 8.0, 5mM EDTA pH 8.0, and 0.1% SDS for 20 min at 50°C.

10 The wash solution was removed, and substrate solution (100mM HEPES Citrate-phosphate buffer (pH 6.5), 90μM 3-methyl-2-benzothiazolinone hydrazone, 6mM 4-methoxynaphthol, and 4mM H<sub>2</sub>O<sub>2</sub>, or 19mM 9-aminoethylcarbazol, 100mM NaOAc pH 4.5, and 7mM H<sub>2</sub>O<sub>2</sub>) was

15 added. Hybridized probe was detected as a blue or brown precipitate, respectively, on the *Bacteroides gingivalis* DNA slots.

Fn-1B:alkaline phosphatase or Bg-1B:alkaline phosphatase were hybridized for 1 hour at room temperature to the Nytran membranes as above. The membranes

20 were washed as above and substrate solution (0.6mM nitro-blue tetrazolium in 70% dimethylformamide, 0.6mM 5-bromo-4-chloro-3-indolyl phosphate in 0.1M Tris-HCl pH 9.5, 0.1M NaCl, 50mM MgCl<sub>2</sub>) was added. Hybridized probe was

25 detected as a blue precipitate on *F. nucleatum* or *B. gingivalis* DNA slots, respectively.

Sandwich hybridizations with the Bg-1B:HRP probe was performed as follows. Varying concentrations of Bg-5B:SIAB probe in PBS (150mM NaCl, 10mM sodium phosphate

30 pH 7.01) were immobilized to cysteamine-derivatized Pall Immunoaffinity membranes (Pall Corporation, East Hills, N.Y.). The membranes were made by conjugating Pall Immunoaffinity membranes (0.2 μ pore size) with 10mM cystamine in PBS and then washing and treating the

35 cystamine-derivatized membranes with DTT (10mM for 30 min at 25°C) to produce a cysteamine-derivatized membrane. Non-covalently immobilized probe was removed from these

membranes by heating at 90°C in PBS. The Pall:Bg-5B membranes were hybridized with a DNA target (complementary to Bg-5B) and Bg-1B:HRP (Bg-1B:5'GAATAACGGGCGATACGAGTATTGATTGAATGTACCGTAAGAATAAGCAT CGG 3') for 30 min in 0.6M NaCl, 30% formamide, 90mM Tris-HCl (pH 8.0), 10mM EDTA, 0.1% SDS at 42°C. The complex was washed in 0.09M NaCl, 9mM Tris (pH 8.0), 1mM EDTA at 50°C for 20 min. The wash solution was decanted and the 4-chloro-methoxynaphthol substrate added. After a 15 min incubation, at room temperature, the hybridized Bg-1B:HRP probe was detected as a blue precipitate on the Pall:Bg5B membrane. Purified Bg 16S rRNA hybridized in a like manner also was detected by sandwich hybridization with Bg-1B:HRP and Pall:Bg5B.

### 15 Example 3

Different concentrations of *B. gingivalis* were lysed with 3M GuSCN, 5% formamide, 50mM Tris-HCl (pH 7.6), 10 mM EDTA, 2% sarkosyl, and 1% mercaptoethanol. Pall membranes with 0.1 µg of immobilized Bg-5B oligonucleotide were immersed into the lysate and hybridized for 0.5 to 24 hours at room temperature. The membranes were washed with a solution containing 0.09M NaCl, 9 mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.1% SDS. The Pall:Bg-5B:rRNA membranes were hybridized again in the GuSCN/formamide lysis solution containing 10-100ng/ml biotinylated signal oligonucleotide and hybridized from 0.5 to 24 hours. The biotinylated signal oligonucleotides used were UP7B, UP9A, and UP3A. The membranes were washed with a solution containing 0.09 M NaCl, 9 mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.5% SDS. The membranes were then incubated with the strepavidin conjugate of alkaline phosphatase (or horse-radish peroxidase) in 0.18M NaCl, 18mM Tris-HCl (pH 8.0), 2mM EDTA, 0.5% SDS, and 0.1% gelatin at room temperature for 15 to 60 min. After the incubation the membranes were washed three times for 1 min each with 0.18M NaCl, 18mM

Tris-HCl (pH 8.0), 2mM EDTA, and 0.5% SDS, then two times for 1 min each with 0.18M NaCl, 18mM Tris-HCl (pH 8.0), and 2mM EDTA, and finally once with appropriate substrate buffer. Color was developed for the two enzymes as described in Example 2. The hybridized signal probe was detected as brown and blue spots for alkaline phosphatase and horse radish peroxidase, respectively. Depending on the enzyme and hybridization conditions, detection of  $1 \times 10^7$  B. gingivalis cells was observed. Signal intensity is dependent upon the number of biotinylated signal oligonucleotides used, and is 3X less when only one signal oligonucleotide is present in the assay.

#### Example 4

A test kit for the detection of oral pathogenic bacteria will contain all of the required components for the collection, processing, and evaluation of several samples, as well as the associated instructions and patient data collection cards.

Product Insert. The Product Insert will contain complete written instructions for patient sampling and evaluation. The instructions will follow the procedures of Example 3.

Data Card. A Data Card will be included for the recording of minimal baseline data for each patient, such as patient identification, site of collection, and test results.

Currettes. Currettes for sampling by scraping.

Endodontic Points. Endodontic points (paper points) for collection of each sample to be tested are also included. After cleansing the supragingival surfaces by wiping with gauze, the point will be used to rub the bacteria from the subgingival surface of the tooth to be sampled and to



collect bacteria by absorption of saliva, gingival fluid, and gingival plaque.

Lysing Reagent. Each point with the collected sample will be placed immediately into a numbered tube of Lysing  
5 Reagent which will lyse the bacteria and release the bacterial nucleic acids.

Probe/Enzyme Reagent. A standard aliquot of Probe labeled by a ligand with or directly conjugated to an Enzyme Reagent is added to each tube of Lysing Reagent to  
10 initiate the hybridization reaction between the pathogen nucleic acid targets and the signal oligonucleotide probes derived from conserved regions of the ribosomal RNA sequences.

Dipstick Device. An individual Dipstick Device  
15 containing site(s) with pathogen-specific DNA probes covalently immobilized to the solid support and having space for marking and identifying each site tooth sampled, is inserted immediately into each tube containing the hybridization mixture and incubated at room temper-  
20 ature.

Wash Reagent. Each Dipstick Device is removed from the hybridization mixture and washed with the Wash Reagent, using the bottle provided.

Enzyme Substrate Reagent. The Dipstick Devices are  
25 placed collectively into the Enzyme Substrate Reagent bottle and developed for several minutes to 1 hour at room temperature. Each Dipstick Device is washed again with the Wash Reagent to remove excess background color.

Reference Card. Color development is visualized and  
30 compared with a Reference Card, indicating the pathogens providing a positive signal.

Table 1. Oligonucleotides specific for bacteria associated with periodontal disease.

		Oligonucleotide Primers Used to Derive Sequence
5	<u>Bacteroides gingivalis</u>	
	Bg-1B 5'CAATACTCGTATCGCCCGTTATTC3'	UP4B/1B
	Bg-4B 5'GTATAAAAGAAGTTTAMAATCCTT3'	UP4B/1B
	Bg-6B 5'CCTTAGGACAGTCTTCCTTCACGC3'	UP4B/1B
10	Bg-3B 5'CTGTGGAAGCTTGACGGTATATCG3'	UP2B
	Bg-8B 5'GGTTTTTCACCATCAGTCATCTACA3'	UP8B
	Bg-7B 5'ATTACCCTAGTGCGCCCTTGCGG3'	UP6B
	Bg-5B 5'CCGATGCTTATTCTTACGGTACAT3'	UP4B/1B
15	<u>Actinobacillus (ex. Haemophilus) actinomycetemcomitans</u>	
	Aa-3B 5'ATTTAACGTCAATTTGGCATGCTA3'	UP4B/1B
	Aa-2B 5'CTTCGGGCACYAGGGCTAAACCCC3'	UP2B
	Aa-4B 5'ACCCATCTCTGAGTTCTTCTTCGG3'	UP8B
	Aa-5B 5'GTGGTAAACGCCCCCTCTCGGTT3'	UP6B
20	Aa-10B 5'TGGCATGCTATTAACACACCAACC3'	UP4B/1B
	<u>Bacteroides intermedius - Type I</u>	
	Bi-1B 5'GGTCCTTATTCTGAAGGGTAAATGC3'	UP4B/1B
	Bi-3B 5'CACGTGCCCCACTTTACTCCCCAA3'	UP4B/1B
	Bi-6B 5'TAGCCGCTAACGCCAGGCGCTAAC3'	UP2B
25	Bi-2B 5'CCCTAGGYGCGCTCCTCGCGGTTA3'	UP6B
	Bi-5B 5'GAGTCAACATCTCTGTATCCTGCG3'	UP8B
	Bi-4B*5'TTGCCCTAGGTCGCTCCTCGCGGT3'	UP6B
30	*Designed to hybridize to both Type I and II, Note Overlaps with Bi-2B	
	<u>Bacteroides intermedius - Type II</u>	
	2Bi-3B 5'AGACGCCCCGAAGGAAGCCTATGT3'	UP8B
	2Bi-1B 5'ATGAGGTACATGCAATGGCGCACAA3'	UP4B
	2Bi-2B 5'CGTGCGCCAATTTATTCCCACATA3'	UP4B
35	<u>Bacteroides forsythus</u>	
	Bf-1B 5'CACAAGGTACATGCAATAAAATAC3'	UP4B
	Bf-2B 5'CGTATCTCATTTTATTCCCCTGTA3'	UP4B
	Bf-5B 5'AGCTCTCACTCTCCGTCGTCTACA3'	UP4B
	Bf-6B 5'AATACACGTATCTCATTTTATTCC3'	UP4B
40	Bf-3B 5'GAAGAAAGCTCTCACTCTCCGTCG3'	UP8B
	Bf-4B 5'CTGTAGAGCTTACACTATATCGCA3'	UP2B

Eikenella corrodens

	Eik-4B	5'GTACGCTACTAAGCAATCAAGTTG3'	UP2B
	Eik-1B	5'TTAGGTACCGTCAGCAAAAAGTGG3'	UP4B
	Eik-2B	5'GCACTTCCCTTTTCTTCCCTAACA3'	UP4
5	Eik-3B	5'TACCGTGGCAAGCGGGCTCCTTGC3'	UP6B
	Eik-5B	5'CTTCCGTCTCTGGAAGGTTCCGTAC3'	UP8B

Fusobacterium nucleatum/periodonticum

	Fn-1B	5'GTCATCGTGCACACAGAATTGCTG3'	UP7B
10	Fn-2B	5'GTTGGTACCGTCATTTTTTTCTTC3'	UP4B
	Fn-3B	5'AGGTTTCCCCGAAGGCACTGAAAC3'	UP8B
	Fn-4B	5'TCAGACTCTCGGTCCATTGTCCAA3'	UP4B/1B
	Fn-6B	5'AAACATCTCTGTCTCATTCTAAG3'	UP8B

Wolinella recta

15	Wr-1B	5'GTACCGTCATAATTCTTTCCCAAG3'	UP4B
	Wr-2B	5'GGACCATAACCGGTTTGGTATTG3'	UP6B
	Wr-3B	5'GCATTACTGCCTCGACTAGCGAAG3'	UP2B
	Wr-6B	5'CTTGGGTACCGTCATAATTCTTTCC3'	UP4B

Streptococcus intermedius

20	Si-1B	5'GTACCGTCACAGTATGAACTTTCC3'	UP4B
	Si-2B	5'TTCTCACACTCGTTCTTCCTTAAC3'	UP4B
	Si-3B	5'TTCCATTCTCACACTCGTTCTTC3'	UP4B

Table 2. Universal Primers for Sequencing and Conserved Signal Oligonucleotides.

<u>16S rRNA oligonucleotide probes</u>		<u>E. coli base position</u>
5	UP1B* 5'GWATTACCGCGGCKGCTG3'	519-536
	UP4B 5'GCTGGCACGGAGTTAGCCG3'	504-522
	UP7B 5'GTATTACCGCGGCTGCTG3'	519-536
	UP2B* 5'CCGTCAATTCATTTAAGTTT3'	907-926
	UP2D 5'CCCGTCWATTCMTTGTAGTTTT3'	906-927
10	UP2A 5'CCCGTCAATTCATTTGAGTTTT3'	906-927
	UP3B* 5'ACGGGCGGTGTGTRC3'	1392-1406
	UP3A 5'TGACGGGCGGTGTGTACAA3'	1390-1408
	UP9A 5'CTGCTGCCTCCCGTAGGAGT3'	338-357
	UP15B 5'GGGTATCTAATCCKGTTYGCTCC3'	775-797
15	UP20B 5'GACTACYMGGGTATCTAATCC3'	785-805
	UP21A 5'TTAAACCACATGYTCCWCCGCTTG3'	936-959
<u>23S rRNA oligonucleotide probes</u>		<u>E. coli base position</u>
20	UP12B 5'TYGATTGGCMTTTCACCCC3'	775-793
	UP13B 5'GGGGTTCTTTTCGCCTTTCC3'	468-487
	23UPA 5'CTTAGATGCTTTCAGC3'	2744-2759
	23UPB 5'CCGGTCCTCTCGTACTA3'	2653-2669
	23UPC 5'GGACCGAACTGTCTCACGACGTTCT3'	2587-2611
	23UPD 5'GACCGCCCCAGTCAAAC3'	2241-2258
	23UPE 5'CCCGACAAGGAATTCGCG3'	1933-1950
	23UPF 5'CCTTCTCCCGAAGTTACGG3'	1685-1703
	23UPG 5'GACCAGTGAGCTATTACGC3'	1091-1109
	23UPH 5'TTCGGGGAGAACCAGCTA3'	803-820
25	23UPJ 5'TTCGCTCGCCGCTACT3'	241-256
	23UPM 5'GTTATAGTTACGGCCGCCGTTTAC3'	1897-1920

\* Equivalent to ribosomal RNA sequencing primers described by Lane et al.

Table 3. Bacteria Specific Oligonucleotide Probes Derived from 23S Ribosomal RNA Sequences.

		Oligonucleotide Primers Used to Derive Sequence
5	<u>Bacteroides gingivalis</u>	
	Bg23-1 5'TTGGGAGCCGGTTTACATCCTTAT3'	23UPF
	Bg23-2 5'GTACGGGTAACACAGAAATATGCT3'	23UPF
	Bg23-3 5'CTCAAATTGCTTTTTTTGATAGCTT3'	23UPE
10	Bg23-4 5'GACTATATACCTCAAATTGCTTTT3'	23UPE
	Bg23-5 5'TTCTCTTGACGATGACTCCTCCTC3'	23UPE
	Bg23-6 5'CCTACACATCTGATGCCAAATACA3'	23UPD
	<u>Fusobacterium nucleatum</u>	
	Fn23-1 5'TCGATTAAGACTCCATCTTAATAG3'	23UPJ



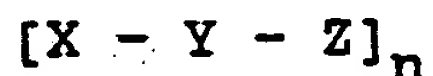
Table 4. Probes Derived from Hypervariable and Conserved Regions of the 16S and 23S Ribosomal RNA which are Free of Secondary and Tertiary Interactions.

<u>Oligonucleotide probe</u>		<u>E. coli base position</u>
5	16S rRNA Hypervariable Regions	
	Aa-4B 5'ACCCATCTCTGAGTTCTTCTTCGG3'	990-1030
	Aa-10B 5'TGGCATGCTATTAACACACCAACC3'	445-475
	Bg-6B 5'CCTTAGGACAGTCTTCCTTCACGC3'	395-430
	Bg-8B 5'GGTTTTTCACCATCAGTCATCTACA3'	990-1030
10	Bg-5B 5'CCGATGCTTATTCTTACGGTACAT3'	475-505
	Bi-3B 5'CACGTGCCCCACTTTACTCCCCAA3'	445-475
	Bi-5B 5'GAGTCAACATCTCTGTATCCTGCG3'	990-1030
	2Bi-2B 5'CGTGCGCCAATTTATTCCACATA3'	445-475
	Eik-4B 5'GTACGCTACTAAGCAATCAAGTTG3'	828-865
15	Eik-2B 5'GCACTTCCCTTTTCTTCCCTAACAA3'	445-475
	Eik-5B 5'CTTCCGTCTCTGGAAGGTTCCGTAC3'	990-1030
	Fn-2B 5'GTTGGTACCGTCATTTTTTTCTTC3'	445-475
	Fn-4B 5'TCAGACTCTCGGTCCATTGTCCAA3'	445-475
	Fn-6B 5'AAACATCTCTGTCTCATTCCTAAG3'	990-1030
20	Wr-1B 5'GTACCGTCATAATTCTTTCCCAAG3'	445-475
	Wr-6B 5'CTTGGGTACCGTCATAATTCTTTCC3'	445-475
	23S rRNA Hypervariable Regions	
	Bg23-2 5'GTACGGGTAACACAGAAATATGCT3'	1570-1620
	Bg23-4 5'GACTATATACCTCAAATTGCTTTT3'	1800-1830
25	Bg23-6 5'CCTACACATCTGATGCCAAATACA3'	2085-2120
	16S rRNA Conserved Regions	
	UP2D 5'CCCGTCWATTCMTTGTAGTTTT3'	906-927
	UP3A 5'TGACGGGCGGTGTGTACAA3'	1390-1408
	UP7B 5'GTATTACCGCGGCTGCTG3'	519-536
30	UP9A 5'CTGCTGCCTCCCGTAGGAGT3'	338-357
	UP20B 5'GACTACYMGGGTATCTAATCC3'	785-805
	UP21A 5'TTAAACCACATGYTCCWCCGCTTG3'	936-959
	23S rRNA Conserved Regions	
	UP12B 5'TYGATTGGCMTTTCACCCC3'	775-793
35	23UPB 5'CCGGTCCTCTCGTACTA3'	2653-2669
	23UPJ 5'TTCGCTCGCCGCTACT3'	241-256
	23UPM 5'GTTATAGTTACGGCCGCGTTTAC3'	1897-1920

## WHAT IS CLAIMED IS:

1. A composition of polynucleotide probes for the detection of bacteria associated with a human oral medical disorder, wherein said probes comprise a segment of nucleic acid capable of selectively hybridizing, under hybridizing conditions, to hypervariable regions of ribosomal RNA of the bacteria with the provision that any additional nucleotides covalently bound to said segment do not hybridize under said conditions to nucleic acids of bacteria found in the human mouth.

2. A polynucleotide probe of the formula:



wherein:

a) X is a sequence of 0 to 100 nucleotides or nucleotide analogs that are non-homologous to regions of bacterial nucleic acid found in bacteria inhabiting human mouths;

b) Y is a sequence of 10 to 100 nucleotides or nucleotide analogs that are capable of hybridizing under hybridizing conditions to hypervariable regions of the ribosomal RNA of bacteria inhabiting human mouths such that Y may also comprise subsequences that are capable of hybridizing under hybridizing conditions to only one species of said bacteria to two species of said bacteria or to three species of said bacteria;

c) Z is a sequence of nucleotides the same as or different from X such that nucleotides or nucleotide analogs are non-homologous to nucleic acid of bacteria inhabiting human mouths; and

d) n is 1-500 and, where n is greater than 1, Y can be the same or different sequences of nucleotides having said hybridization capability.

3. A composition according to claim 1 wherein said bacteria is selected from the group consisting of Actinobacillus (ex. Haemophilus) actinomycetemcomitans, Bacteroides gingivalis, Bacteroides intermedius Type 1, Bacteroides intermedius Type 2, Eikenella corrodens, Bacteroides forsythus, Fusobacterium nucleatum, Fusobacterium periodonticum, Streptococcus intermedius, Wolinella recta, and combinations thereof.

4. A composition according to claim 1 wherein the ribosomal RNA is selected from the group of ribosomal RNA having a sedimentation coefficient of 16S or 23S.

5. A composition according to claim 1 wherein probes comprise from 15 to 50 nucleotides.

6. A composition according to claim 3 wherein said bacteria are Bacteroides gingivalis.

7. A composition according to claim 6 wherein said nucleic acid segment is selected from the group consisting of:

5'CAATACTCGTATCGCCCGTTATTC3';  
5'GTATAAAAGAAGTTTAMAATCCTT3';  
5'CCTTAGGACAGTCTTCCTTCACGC3';  
5'CTGTGGAAGCTTGACGGTATATCG3';  
5'GGTTTTTCACCATCAGTCATCTACA3';  
5'ATTACCCTAGTGCGCCCCTTGCGG3';  
5'CCGATGCTTATTCTTACGGTACAT3';

and combinations thereof.

8. A composition according to claim 3 wherein said bacteria are Actinobacillus (ex. Haemophilus) actinomycetemcomitans.

9. A composition according to claim 8 wherein said oligonucleotide sequence is selected from the group consisting of:

5  
5'ATTTAACGTCAATTTGGCATGCTA3';  
5'CTTCGGGCACYAGGGCTAAACCCC3';  
5'ACCCATCTCTGAGTTCTTCTTCGG3';  
5'GTGGTAAACGCCCCCTCTCGGTT3';  
5'TGGCATGCTATTAACACACCAACC3';

and combinations thereof.

10. A composition according to claim 3 wherein said bacteria are *Bacteroides intermedius* Type 1.

11. A composition according to claim 10 wherein said nucleic acid segment is selected from the group consisting of:

15  
5'GGTCCTTATTCGAAGGGTAAATGC3';  
5'CACGTGCCCCACTTTACTCCCCAA3';  
5'TAGCCGCTAACGCCAGGCGCTAAC3';  
5'CCCTAGGYGCGCTCCTCGCGGTTA3';  
5'GAGTCAACATCTCTGTATCCTGCG3';  
20  
5'TTGCCCTAGGTCGCTCCTCGCGGT3';

and combinations thereof.

12. A composition according to claim 3 wherein said bacteria are *Bacteroides intermedius* Type 2.

13. A composition according to claim 12 wherein said nucleic acid segment is selected from the group consisting of:

25  
5'AGACGCCCCGAAGGAAGCCTATGT3';  
5'ATGAGGTACATGCAATGGCGCACA3';  
5'CGTGCGCCAATTTATTCCCACATA3';

30 and combinations thereof.

14. A composition according to claim 3 wherein said bacteria are *Bacteroides forsythus*.

15. A composition according to claim 14 wherein said nucleic acid segment is selected from the group consisting of:

5  
5' CACAAGGTACATGCAATAAAATAC3';  
5' CGTATCTCATTTTATTCCCCTGTA3';  
5' AGCTCTCACTCTCCGTCGTCTACA3';  
5' AATACACGTATCTCATTTTATTCC3';  
5' GAAGAAAGCTCTCACTCTCCGTCG3';  
5' CTGTAGAGCTTACACTATATCGCA3';

10 and combinations thereof.

16. A composition according to claim 3 wherein said bacteria are *Eikenella corrodens*.

17. A composition according to claim 16 wherein said nucleic acid segment is selected from the group  
15 consisting of:

5' GTACGCTACTAAGCAATCAAGTTG3';  
5' TTAGGTACCGTCAGCAAAAAGTGG3';  
5' GCACTTCCCTTTTCTTCCCTAACA3';  
5' TACCGTGGCAAGCGGGCTCCTTGC3';  
20 5' CTTCCGTCTCTGGAAGGTTCCGTAC3';

and combinations thereof.

18. A composition according to claim 3 wherein said bacteria are *Fusobacterium nucleatum*.



19. A composition according to claim 18 wherein said nucleic acid segment is selected from the group consisting of:

5  
5'GTCATCGTGCACACAGAATTGCTG3';  
5'GTTGGTACCGTCATTTTTTTCTTC3';  
5'AGGTTTCCCCGAAGGCACTGAAAC3';  
5'TCAGACTCTCGGTCCATTGTCCAA3';  
5'AAACATCTCTGTCTCATTCTAAG3';

and combinations thereof.

10           20. A composition according to claim 3 wherein said bacteria are *Wolinella recta*.

21. A composition according to claim 20 wherein said nucleic acid segment is selected from the group consisting of:

15  
5'GTACCGTCATAATTCTTTCCCAAG3';  
5'GGACCATAACCGGTTTGGTATTG3';  
5'GCATTACTGCCTCGACTAGCGAAG3';  
5'CTTGGGTACCGTCATAATTCTTTCC3';

and combinations thereof.

20           22. A composition according to claim 3 wherein said bacteria are *Streptococcus intermedius*.

23. A composition according to claim 22 wherein said nucleic acid segment is selected from the group consisting of:

25  
5'GTACCGTCACAGTATGAACTTTCC3';  
5'TTCTCACACTCGTTCTTCCTTAAC3';and  
5'TTCCATTCTCACACTCGTTCTTC3'.

24. A method of detecting, in a sample obtained from the mouth of a human patient, bacteria associated with a medical disorder, said method comprising the steps of:

5 lysing the microbial cells to free the ribosomal RNA in said sample;

contacting said ribosomal RNA, under hybridizing conditions, with polynucleotide probes capable of selectively hybridizing to the hypervariable regions of the  
10 ribosomal RNA of said bacteria; and

detecting hybridization complexes as an indication of the presence of the microbial cell in the sample.

25. A method according to claim 24 wherein said  
15 probe is capable of hybridizing to the ribosomal RNA of bacteria selected from the group consisting of Actinobacillus (ex. Haemophilus) actinomycetemcomitans, Bacteroides gingivalis, B. intermedius Type 1, Bacteroides intermedius Type 2, Eikenella corrodens,  
20 Bacteroides forsythus, Fusobacterium nucleatum, Fusobacterium periodonticum, Streptococcus intermedius, and Wolinella recta and combinations thereof.

26. A method according to claim 25 wherein the  
25 sample is assayed using at least two different groups of probes where each group is capable of hybridizing to a different hypervariable region of the ribosomal RNA of a single bacterium.

27. A diagnostic kit for use in determining the  
30 presence of a specific polynucleotide sequence which comprises a synthetic oligonucleotide probe complementary to the hypervariable region of the ribosomal RNA of bacteria associated with a human oral medical disorder.

28. A diagnostic kit according to claim 27, further comprising a lysing reagent, probe/enzyme reagent, dipstick device, wash reagent, enzyme substrate reagent, and a reference card.

5           29. A composition of polynucleotide probes for the detection of bacteria associated with a human oral medical disorder, wherein said probes comprise a segment of nucleic acid capable of selectively hybridizing, under hybridizing conditions, to open regions of ribosomal RNA  
10 of the bacteria having minimal secondary or tertiary interactions with adjacent nucleotides, said probes substantially binding only to open regions.

          30. A composition according to claim 29 wherein the probes selectively hybridize to conserved regions of  
15 ribosomal RNA.

          31. A composition according to claim 30 wherein said nucleic acid segment is selected from the group consisting of:

                  5'CCCGTCWATTCMTTGTGAGTTTT3';  
20                   5'TGACGGGCGGTGTGTACAA3';  
                  5'GTATTACCGCGGCTGCTG3';  
                  5'CTGCTGCCTCCCGTAGGAGT3';  
                  5'GACTACYMGGGTATCTAATCC3';  
                  5'TTAAACCACATGYTCCWCCGCTTG3';  
25                   5'TYGATTGGCMTTTCACCCC3';  
                  5'CCGGTCCTCTCGTACTA3';  
                  5'TTCGCTCGCCGCTACT3';  
                  5'GTTATAGTTACGGCCGCGGTTTAC3';

and combinations thereof.

30           32. A composition according to claim 29 wherein the probes selectively hybridize to hypervariable regions of ribosomal RNA.

33. A composition according to claim 32 wherein said nucleic acid segment is selected from the group consisting of:

5  
5'ACCCATCTCTGAGTTCTTCTTCGG3';  
5'TGGCATGCTATTAACACACCAACC3';  
5'CCTTAGGACAGTCTTCCTTCACGC3';  
5'GGTTTTTCACCATCAGTCATCTACA3';  
5'CCGATGCTTATTCTTACGGTACAT3';  
5'CACGTGCCCCACTTTACTCCCCAA3';  
10 5'GAGTCAACATCTCTGTATCCTGCG3';  
5'CGTGCGCCAATTTATTCCCACATA3';  
5'GTACGCTACTAAGCAATCAAGTTG3';  
5'GCACTTCCCTTTTCTTCCCTAACA3';  
5'CTTCCGTCTCTGGAAGGTCCGTAC3';  
15 5'GTTGGTACCGTCATTTTTTTCTTC3';  
5'TCAGACTCTCGGTCCATTGTCCAA3';  
5'AAACATCTCTGTCTCATTCTAAG3';  
5'GTACCGTCATAATTCTTTCCCAAG3';  
5'CTTGGGTACCGTCATAATTCTTTCC3';  
20 5'GTACGGGTAACACAGAAATATGCT3';  
5'GACTATATACCTCAAATTGCTTTT3';  
5'CCTACACATCTGATGCCAAATACA3';

and combinations thereof.

34. A method of detecting, in a sample obtained  
25 from the mouth of a human patient, bacteria associated with a medical disorder, said method comprising the steps of:

lysing the microbial cells to free the ribosomal RNA in said sample;

30 contacting said ribosomal RNA, under hybridizing conditions, with polynucleotide probes capable of selectively hybridizing to open regions of the ribosomal RNA having minimal secondary and tertiary interactions with adjacent nucleotides; and

35 detecting hybridization complexes as an indi-

cation of the presence of the microbial cell in the sample.

35. A method according to claim 34 wherein said probe is capable of hybridizing to the ribosomal RNA of bacteria selected from the group consisting of Actinobacillus (ex. Haemophilus) actinomycetemcomitans, Bacteroides gingivalis, B. intermedius Type 1, Bacteroides intermedius Type 2, Eikenella corrodens, Bacteroides forsythus, Fusobacterium nucleatum, Fusobacterium periodonticum, Streptococcus intermedius, and Wolinella recta and combinations thereof.

36. A method according to claim 35 wherein the sample is assayed using at least two different groups of probes where each group is capable of hybridizing to a different conserved region of the ribosomal RNA of a single bacterium.

37. A composition of polynucleotide probes for the detection of bacteria associated with a human oral medical disorder, wherein said probes comprise a segment of nucleic acid capable of selectively hybridizing, under hybridizing conditions, to conserved regions of ribosomal RNA of the bacteria with the provision that any additional nucleotides covalently bound to said segment do not hybridize under said conditions to nucleic acids of bacteria found in the human mouth.



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10

15

and combinations thereof.

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/00072**

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

**IPC(4): C12Q 1/70**

**U.S. Cl.: 435/6**

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System

Classification Symbols

**U.S. Cl.**

**435/6, 7  
436/501, 63, 27**

**935/2, 19, 20, 21, 78**

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category *	Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	EP, A, 144,914, Alberetta, 12 June 1985	1-38
Y	EP, A, 209,702, Carrico, 28 January 1987	1-38
Y	EP, A, 133,671, Deriemer, 06 March 1985	1-38

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

**07 April 1989**

International Searching Authority

**ISA/US**

Date of Mailing of this International Search Report

**12 JUN 1989**

Signature of Authorized Officer

*Amelia Burgess Yarbrough*  
**Amelia Burgess Yarbrough**